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Environmental Significance Statement

While the antimicrobial activity of ionizing nanoparticles is largely attributed to the released ions, the role of the particle is not yet fully realized. This mechanistic ambiguity is not only due to complex exposure environments, but also inconsistencies in experimental designs, the most critical being the ion controls. We identify studies that scale Ag(I) ion controls to the concentration of Ag(I) released from AgNPs and find particle-specific effects acting jointly with and/or sometimes independently from the ions. This support for the particle suggests the ability to leverage manipulation of particle properties to further tune the mechanism and magnitude of AgNP antimicrobial activity. This strategic design of nano-enabled antimicrobial agents could positively impact global public health in this era of antimicrobial resistance.

Emerging investigator series: It's Not All About the Ion: Support for Particle-Specific Contributions to Silver Nanoparticle Antimicrobial Activity

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Abstract

Silver nanoparticles (AgNPs) and other ionizing engineered nanomaterials (ENMs) are candidates for the development of antimicrobial agents due to their efficacy, multiple modes of bacterial inactivation, and tunability with respect to both the magnitude and mechanisms of antimicrobial activity. Exploiting this versatility requires elucidating the bacterial inactivation pathway(s) of the ENM, and in particular, the link between material properties and the desired biological endpoint. The mechanisms of antimicrobial activity for macrosilver, silver salts, and AgNPs have been widely studied, and largely attribute this activity to the release of Ag ions via oxidation and dissolution of the surface Ag atoms. However, it has also been established that Ag ion exposure alone does not elicit the same bacterial response as exposure to AgNPs, which suggests that the observed antimicrobial activity is induced not only by solubilized ions but also by the ENM itself. Resolving the role of the AgNP is critical to informing design of nano-enabled antimicrobials *a priori*. Herein, we present a systematic review of the AgNP antimicrobial activity literature, and specifically focus on studies that scale Ag ion controls to the likely quantities of bioavailable Ag released from AgNPs. This literature selection criterion reveals the critical role of scaled ion controls in distinguishing ion and particle contributions to the observed antimicrobial activity. Overall, our analysis of this literature indicates that in most cases of bacteria exposure to AgNPs, particle-specific activity is observed and acts in concert with and/or independently from solubilized Ag ions alone. These results are exciting and suggest that more efficacious Ag- and ENM-enabled antimicrobials can be obtained through ENM design.

1. INTRODUCTION

Antimicrobial agents are crucial and ubiquitous in many industries, including health care,[1-9] food and agriculture,[6, 7, 10-12] water treatment,[4, 6-8, 13] and drinking water distribution.[4, 6-8, 13] An increasingly critical challenge associated with antimicrobial use is that the target microbe can build resistance over time.[1, 3, 4, 6, 9, 14-18] Silver nanoparticles (AgNPs) and other ionizing engineered nanomaterials (ENMs) are candidates for the development of superior antimicrobial agents due to their efficacy, multiple modes of organism inactivation, and tunability with respect to both the magnitude and mechanisms of antimicrobial activity. Exploiting the enhanced functionality of ENMs as well as ensuring that they combat rather than contribute to the global antimicrobial resistance challenge requires resolving their mechanisms of organism inactivation, particularly the link between material properties and the desired biological endpoint.

Silver and AgNPs are among the oldest and most widely used antimicrobial agents,[13, 19, 20] and their antimicrobial properties have been studied extensively.[14, 18] For example, dating back to 7,000 years ago, silver-lined vessels and silver coins in containers of water or milk were used to preserve rations during military conflicts.[9, 21] Silver has also been used to treat ulcers and aid in wound healing.[14, 21] The antimicrobial mechanism(s) of silver at the macroscale are attributed to the release of Ag(I) ions and their interactions with various aspects of the microbe.[14, 18, 21] For example, Ag(I) ions can interact with sulfhydryl groups on the cell surface, where the subsequent formation of the Ag-S bonds blocks respiration and electron transfer, leading to the collapse of the proton motive force, the de-energizing of the membrane, and eventually cell death.[4] The ionic radius of a Ag(I) ion is also sufficiently small (0.115 nm)[22] to

travel through transmembrane proteins such as porins (30-50 kDa; pore size, 1- 3 nm).[23] Once inside the cell, Ag(I) ions may react with thiol functional groups in proteins and nucleic acids, interfering with DNA replication and deactivating many enzymatic functions.[4] In general, Ag(I) ions also increase the level of reactive oxygen species (ROS) inside the cell because thiol-containing anti-oxidative enzymes are deactivated by silver, thus exacerbating the damage done to proteins, lipids, and nucleic acids.[4]

Compared to their macroscale counterparts, AgNPs exhibit enhanced ion release per unit mass mainly due to an increased surface area to volume ratio. Yet, the precise mechanism(s) of AgNP action remain unresolved, particularly the dynamic contributions of the NP and Ag(I) ion.[14, 18] There are three overarching possibilities for mechanisms of AgNP antimicrobial activity. The first is that AgNPs are simply a passive Ag(I) ion reservoir, and (as in the case of macroscopic silver) released Ag(I) ions are responsible for any antimicrobial activity.[14, 24-30] The second possibility is that antimicrobial activity of AgNPs is a result of particle-only effects (e.g., physical disruption or alteration of the phospholipid cell membrane, production of ROS at the cell surface, or surface proteins that can bind to the NP but do not bind Ag(I) ions), putting into question the necessity of Ag(I) ions and their claim as the main agent of cellular impact.[14, 24, 31, 32] The third possibility is some combination of the first two mechanisms, and/or some degree of synergistic effects between the released Ag(I) ions and the particle action. For example, there may be unique passive transport pathways in the bacterial cell (e.g., outer membrane porins, altered membrane permeability) accessible to particles at the nanoscale that facilitate the delivery of intracellular Ag(I) ions, a phenomenon known as the Trojan horse mechanism.[14, 28, 33-36 37] Overall, the current literature contains support for all

three possibilities. This ambiguity in the mechanisms of AgNP antimicrobial activity limits our ability to rationally design nanoparticle-enabled antimicrobials, and further, to leverage the material tunability of these ENMs.

While some of the existing mechanistic ambiguity is surely due to the complexity of the systems themselves, a non-trivial component arises from inconsistences in experimental designs across studies. Critical components of the experimental design include control of AgNP morphology (i.e. size, shape, surface chemistry), NP synthesis and washing steps, composition of the bacterial growth medium, bacterial strain used, measured toxicity endpoint and the methodology used to assess it,[38] and the treatment of the pure Ag(I) ion control.

Of all of these parameters, the most critical for distinguishing particle-specific antimicrobial activity is arguably the measurement and experimental controls for Ag(I) ion release. Ion controls are used in experiments analyzing the antimicrobial activity of ionizing ENMs in order to identify the biological effects that arise solely due to the ion portion of the ENM system and then compare to the effects of the ENM. Ion controls may be delivered to the experimental system as a silver salt (e.g., $AgNO_3$, $AgC_2H_3O_2$, Ag_2SO_4) or as the isolated ions in the supernatant of a AgNP suspension. There are two key components to a pure Ag(I) ion control: the concentration of ions introduced to the bacteria and the kinetics of that introduction. The total concentration of Ag(I) ion used as a control should reflect, as closely as possible, the concentration of ions released from the NPs used in the study. Matching these concentrations requires the ability to measure released Ag(I) ions, which can be challenging (*vide infra*).

In order to accurately dose the bacteria with the established concentration of ions, ideally, the rate of Ag(I) ion release would also be known. Many studies deliver a single or pulse dose of Ag(I) ions that is equivalent to the total silver concentration in the AgNP system, which mimics the complete and instantaneous dissolution of the AgNP rather than the release of Ag(I) ion from the AgNP over time. In addition to not capturing the kinetics of Ag(I) ion release, this approach to ion controls does not accurately represent the AgNP system, since only a fraction of the AgNP forms Ag(I) ions at any given time.[39-41] As such, the resulting conclusions regarding ion-only, particle-specific, and combined ion-particle effects are confounded by the inaccurate representation of the Ag(I) ion component of the AgNP system.

Here, we review more than 300 publications on the cytotoxicity of AgNPs, 59 of which specifically aimed to distinguish ion-only, particle-only, and combined ion-particle contributions to the observed antimicrobial activity using methodologies that included an ion control. In other words, we focus on studies that specifically draw conclusions about the *contribution* that the ion and particle play in the *mechanism* of antimicrobial activity and not studies that only evaluated the *potency* of AgNPs. We then critically analyze the conclusions from 30 of these 59 studies (51%), focusing on those studies that implemented scaled ion controls *(vide infra),* to identify trends in particle parameters and other experimental factors that indicate ion-only, particle-specific, and combined ion-particle mechanisms of antimicrobial activity. Interestingly, while the results of this analysis suggest an important role of Ag(I) ions, they also clearly highlight that the impact of the particle alone cannot be ignored. Further, the analysis reveals a critical opportunity to elucidate these particle-specific antimicrobial mechanisms as well as whether particle-specific parameters can be manipulated to influence these mechanisms.

2. STATE OF THE ART: Factors influencing ion release and current practices of scaling ion controls

It is critical to systematically quantify released Ag(I) ions from the AgNPs to inform conclusions regarding the ion and particle contributions on antimicrobial activity. In this section, we discuss the state of the art in measuring the quantity and kinetics of Ag(I) ion release from AgNPs to inform ion controls.

2.1 Factors that influence the extent of AgNP oxidation and ion release can guide ion control selection

For AgNPs in aqueous systems, Ag(I) ion release typically begins with oxidation of the NP surface. Oxidation of the AgNP surface and release of Ag(I) ions involves several processes that occur simultaneously and are dependent on both particle (e.g., size, shape, and surface chemistry) and experimental factors (e.g., dissolved oxygen, time, and broth chemistry).[27, 28, 31, 38, 39, 42-47] Modulating these pathways can control both the quantity and kinetics of ion release.

In general, Ag(I) ion release is initiated by the adsorption of oxygen onto the particle surface followed by subsequent electron transfer.[47] Then, AgNPs evolve a surface bound silver oxide (Ag_2O) layer.[39, 48] The process of ion release begins as this layer is stripped and a new layer forms. However, once the initial silver oxide surface layers are removed by dissolution, Ag(I) ion release is minimized.[39] The amount and strength of oxidizers (e.g., H_2O_2 versus O_2) present in solution influence the extent of oxidation.[40, 49] The stronger the oxidizer (i.e., having greater redox potential), the faster the oxidation rate, where the process follows Arrhenius behavior.[46, 49] Protons are then required for the dissolution of the silver oxide layer and thus, Ag(I) ion release is strongly pH dependent.[39, 46, 48] In addition, suppression of oxidation can

occur with the addition of organic matter and stabilizing ligands, a reduction in temperature, or an increase in pH.[46] Given these differences in kinetics, the relevant extent of Ag(I) ion release also depends on the exposure time between AgNPs and the bacteria system of interest.[46] This time-dependent ion release suggests the importance of monitoring ion release continuously in the AgNP system and using it to inform both the concentration of the ion control and the rate at which it should be delivered to the system in order to mimic the AgNP system as accurately as possible.

The particle alone can influence the oxidation and dissolution process and so the ion control needed for each particle type will be different. Generally, smaller particles have a greater radius of curvature and therefore oxidize at a faster rate, a phenomenon described by the Ostwald-Freundlich equation.[45, 50, 51] Different surface-exposed facets also have different reactivity towards oxygen,[36, 52-55] highlighting the effect of parameters such as particle shape on the oxidation process. Finally, surface chemistry also influences dissolution via capping ligands[33] and insoluble passivation layers, which influence the dissolution behavior (total concentration and location).[42-45, 56] Silver oxide surface layers can be removed to varying degrees when AgNPs are washed post-synthesis (to remove residual impurities), synthesized anaerobically, or synthesized aerobically and reduced by hydrogen to zero-valent AgNPs before exposure to bacteria, which can all significantly reduce Ag(I) release.[39] Biologically synthesized NPs from plant extracts can also induce different surface chemistries (number and packing distribution of ligands and biomolecules) as compared to chemically synthesized NPs.[30, 57, 58] Thus, the AgNP synthesis and purification approach may affect the surface structure and as a result, Ag(I) ion release. These studies highlight the importance of performing and reporting the AgNP method

of synthesis and purification procedures.[31, 39] Overall, particle-type specific dissolution is a determining factor guiding the selection of an appropriately scaled ion control for specific particle types.

It is critical that ion release is monitored in the exposure media/environment of the AgNP system because the type of environment can influence dissolution, and in turn, the ion control that should be used for that specific system. For example, there are multiple types of growth media used to provide vital nutrients that enable bacterial growth (e.g., buffers, sodium chloride, water, and commonly used broths such as Mueller Hinton, MH, and Luria-Bertani, LB). Due to differences in pH and media constituents (e.g., dissolved ionic species, proteins, peptides, carbohydrates), the specific media used can impact dissolution, the magnitude of AgNP antimicrobial activity, and the dominant mechanism through which that activity occurs (i.e., the Ag(I) ion, the AgNP, or a dynamic synergism between the particle and its released Ag(I) ions). In one case, we have compared AgNP antibacterial activity in two commonly used bacterial growth media (LB and MH broth).[38] Using controlled exposures to AgNPs and Ag(I) ion, we measured a differential impact (measured as a difference in the bacterial growth delay and maximum achieved bacterial growth in relation to the untreated bacteria) – in the two media.[38] This difference suggests that there is a complex interplay between the particle and the surrounding environment, which can result in enhancement or inhibition of Ag(I) release from the AgNP surface[42-46, 59] as well as changes in surface charge and particle stability that could eliminate particle-specific effects.[42-45, 49, 60, 61] For example, dissolution can be influenced by the concentration of chloride present in the growth medium; low chloride concentrations form a AgCl(s) passivation layer on the AgNP surface that inhibits dissolution whereas high

concentrations of chloride result in the formation of soluble AgCl complexes and promote dissolution.[59] Overall, the confounding influence of media on AgNP behavior not only cautions comparison of antimicrobial activity across studies, but also underlines the importance of determining interactions of media constituents with themselves, the NP surface, and the released ions.

The presence of bacteria and their metabolic state additionally affect dissolution, and thus will affect the concentration of the ion control needed to accurately model the AgNP system. Bacteria (i) play a role in altering the dissolved oxygen concentration and pH of the experimental system and (ii) introduce extracellular polymeric substances (EPS) that can non-specifically adsorb to AgNPs.[27] For example, EPS (and other components of the growth media) can form a protein corona around the NP surface and either prevent dissolution or increase the dissolution gradient by binding the released Ag(I) ions upon direct association with the cell (via Le Chatelier's principle).[27] In this regard, bulk dissolution should be monitored in the presence of bacteria and be used to inform accurate concentrations and kinetics needed for appropriately scaled ion controls.

Upon release to the experiential system, there are numerous binding and partitioning events that Ag(I) ions can experience. Ag(I) ions can resorb onto the AgNP surface, remain free in solution, complex with media components, bind to the cell surface, or enter the cell where they can bind to intracellular components or be reduced by them to form new AgNPs.[45, 46] However, more work is needed to accurately capture the dynamics of the AgNP oxidation process by considering the complex interplay of all factors discussed.[46] This complexity suggests the need to monitor intracellular ion release in addition to bulk ion release. Also, techniques

should be used to resolve Ag bound to macromolecules in suspension. Combined, such experiments can inform the delivery of ion controls that accurately mimic the AgNP system and allow us to decouple true ion and particle contributions to antimicrobial activity. Figure 1 presents a visual summary of all potential Ag(I) ion release pathways, binding, and partitioning events.

Figure 1

2.2 Equating the concentration of the ion control to the total Ag concentration in the AgNP system

The challenge with many ion controls used in AgNP antimicrobial activity studies is that they are equivalent to the total silver concentration in the AgNP system and therefore, do not accurately represent the portion of Ag(I) ion present in the test system. There are different forms and concentrations of silver present in the AgNP system (i.e., the Ag(0) form of the nanoparticle and the dissolved Ag(I) form, Figure S1A), which influences the bioavailability of the silver and the resulting biological impacts. The amount of Ag(I) present depends on the extent to which the AgNP oxidizes to release Ag(I) ions as well as the tendency for those ions to complex with components of the exposure media (e.g., chloride to form AgCl(s) or soluble AgCl complexes). Typically, only a portion of the AgNP oxidizes to form Ag(I) ions and as a result, the proportion of silver present as Ag(I) is small relative to Ag(0). Therefore, using an ion control at the same mass concentration as the total silver content of the AgNPs (hereafter referred to as an overestimated ion control) makes for an unequal comparison of the resulting biological activity and calls into question the mechanistic conclusions drawn from such studies (Figure S1B-C).

2.3 Quantifying ion release in bulk solution to inform an ion control

An improved approach to selecting the concentration of the ion control involves the quantification of Ag(I) ion release in the AgNP system, which is commonly pursued using either a Ag(I) ion-selective electrode or ultraviolet-visible (UV-vis) spectroscopy, or by first separating the Ag(I) ions from the AgNPs by centrifugation or dialysis and then analyzing with inductively coupled-plasma mass or atomic emission spectroscopy (ICP-MS or AES) or graphite furnace atomic absorption spectroscopy (GF-AAS).[24, 27, 28, 33, 38, 57, 62-71] It is important to note that when dialysis membranes are used to separate ions from particles,[72-74] the osmotic pressure difference does not allow for complete isolation of Ag(I) ions, limiting the utility of this approach. Furthermore, Ag(I) ion concentration in the bulk suspension is most often measured at a single time point (typically at the culmination of the experiment),[24, 27, 28, 33, 62, 64, 65, 70, 72, 75] which excludes the kinetics of ion release. An alternative approach that aims to capture the dynamics of ion release will quantify Ag(I) ion concentration at multiple time points over the duration of the experiment.[26, 38, 57, 63, 66, 68, 73, 76] When this approach is used, Ag(I) ion controls can be employed in a way that closely mimics the AgNP exposure system. Finally, these techniques measure the free Ag(I) ions present in the bulk solution but do not capture the Ag(I) ions that are removed from solution via subsequent interactions with the surrounding environment (e.g., inside the cell or bound to macromolecules in the suspension). As a result of this limitation, the concentration of Ag(I) ions dosed in as a silver salt control may be inconsistent with the "real" concentration released by the AgNP (both intracellularly and extracellularly).

2.4 Quantifying intracellular ion release to inform an ion control

In an effort to circumvent the abovementioned confounding interactions, researchers measure intracellular Ag using a bioluminescent *E. coli* Ag-biosensor[23, 24, 66, 75, 77] or the Ag content of the membrane and cytoplasm fractions of the cell,[33] rationalizing that these fractions of silver are impacting the bacteria. Yet similar to the methods above, intracellular ion release is monitored at a single time point.[24, 66, 75, 77] While intracellular Ag monitoring quantifies the concentration of Ag to which the cell is directly exposed, it has not typically been used to inform the concentrations and dosing of silver salt ion controls. Doing so would allow for accurate modeling of the AgNP system, particularly if used in combination with bulk dissolution monitoring.

2.5 Suggested best-practice for scaling ion controls

The complexity of the system and challenges faced when quantifying the presence of released ions in solution and in the microorganism suggest that a new approach to ion controls is necessary to obtain comprehensive ion release profiles within the AgNP exposure condition. The desired approach will account for the kinetics of Ag(I) ion release and model an accurate representation of the AgNP system. To ascertain and then implement these controls, there are three key experimental components: (i) continuous monitoring of ion release from AgNPs in the exposure media and in the presence of bacteria as well as intracellularly over the duration of the experiment,[26, 38, 57, 63, 66, 68, 73, 76] (ii) deliver these measured ion concentrations as a series of continuous doses that mirror their release from the ENM, and (iii) subsequently monitor the bacterial endpoint of interest. This preferred comprehensive best practice approach to achieve an appropriately scaled ion control, although labor intensive, is necessary to robustly decouple the contributions of the particle and the ion.

3. EXPERIMENTAL

3.1 Literature review

A comprehensive literature survey to identify studies on the cytotoxicity of AgNPs resulted in more than 300 publications. Google Scholar, Scopus, Compendex, and Web of Science databases were queried using all combinations of the following search terms: “silver nanoparticle”, “silver ion”, “antimicrobial”, “(cyto)toxicity”, and “mechanism”. The SciFinder database was also queried using “silver nanoparticle and toxicity and mechanism” as the initial search term and then further refined to include “ion” and “bacteria”. The sheer size of this set of studies and the heterogeneity of experimental designs did not allow for meaningful discernment of the contributions of the AgNP and Ag(I) ions. Given the extensive use of AgNPs as antimicrobials in a wide range of products, we decided to limit the scope of our literature review to studies that use bacteria as their model organism. With the goal of resolving the ion and particle debate, we further narrowed the scope to those publications that specifically aim to distinguish ion-only, particle-only, and combined ion-particle contributions to the observed antimicrobial activity. In other words, we focus on studies that specifically draw conclusions about the *contribution* that the ion and particle play in the *mechanism* of antimicrobial activity and not studies that only evaluated the *potency* of AgNPs (often culminating in the conclusion that the Ag(I) ions are *more toxic* than the AgNPs). This selective literature set included 59 studies. The literature was further refined to attain a final subset of studies that (i) included comprehensive characterization of AgNP size (by transmission electron microscopy, TEM, at a minimum) and shape, (ii) defined the AgNP dose(s) delivered under the exposure condition using ICP-MS, UV-vis, or GF-AAS, and (iii) included a scaled ion control (as determined through

calculation, described in detail below, using results from criteria i and ii) that was delivered under the same exposure conditions as the AgNP (i.e., in the same growth medium and bacteria). This final subset of literature contained 30 studies and was used in our analysis. Given that we used scaled ion controls as a metric for determining inclusion and exclusion of studies (i.e., pre-specified eligibility criteria), we refer to our analysis as a systematic review.[78] A descriptive summary of each study is compiled in Table S1.

A scaled ion control is defined here as having an equivalent concentration of silver atoms to the bioavailable silver that is released from the AgNPs as Ag(I) and is delivered to the experimental system as a silver salt (e.g., $AgNO_3$, $AgC_2H_3O_2$, Ag_2SO_4) or as the isolated ions from the AgNP. This factor is a distinguishing criterion because scaled ion controls are crucial for valid comparison of ion-only, particle-only, and combined ion-particle contributions to AgNP antimicrobial activity. The calculations we use to determine a scaled ion control consider the extent to which a AgNP of a given particle size and shape can dissolve to its bioavailable Ag(I) form based on the surface atoms available for oxidation and will be discussed in detail below.

3.2 Scaled ion control calculations

The following calculations outline our approach to determine scaled ion controls in each experimental AgNP system. Briefly, the percent of AgNP oxidation and subsequent ion release necessary to obtain Ag(I) ion concentrations equivalent to the ion controls used in a given study (i.e., the expected bioavailable silver) was calculated by comparing the total concentration of silver atoms (on a mol/mL basis) present in the reported ion control and in the delivered particle dose(s). The theoretical percentage of AgNP surface atoms available for oxidation was calculated by considering the size and shape of the AgNP studied (*vide infra*), which is why

inclusion of particle characterization by TEM is critical and serves as the basis for defining a scaled ion control.

Due to ambiguity in the concentrations of the ion controls and AgNPs reported in the literature, we carried out the calculations under two feasible assumptions: (i) the reported concentration indicates the concentration of the total silver salt or AgNP, and (ii) the reported concentration indicates the total silver atom concentration of the silver salt or AgNP. The difference in results for these two assumptions was insignificant for the silver salt, but significant for the AgNP. The assumption that the reported concentrations of AgNPs were as amount of AgNPs per unit volume resulted in unrealistic values of necessary AgNP oxidation (e.g., 10^{29}%) and rejection of every study for having an overestimated ion control. Therefore, the calculations proceeded assuming the reported concentration was of the total silver atom concentration unless otherwise specified in the study. This assumption is further rationalized by the fact that those studies including details on how they determined AgNP concentrations used characterization techniques (e.g., ICP-MS or AES, UV-vis, or GF-AAS) that measure total silver atom concentrations.

The following example calculation demonstrates the approach used to determine the concentration of Ag(I) ions in the silver salt control in units of moles per mL:

$$\frac{\mu g}{mL} Ag(I)\ as\ silver\ salt * \frac{1\ g}{10^6\ \mu g} * \frac{1\ mol\ Ag(I)}{107.87\ g} = \frac{mol\ Ag(I)}{mL} as\ silver\ salt \quad (1),$$

which for a reported concentration of 0.4 $\frac{\mu g}{mL}$ Ag(I) (delivered as $AgNO_3$) equates to $3.71x10^{-9}\frac{mol\ Ag(I)}{mL}$(delivered as $AgNO_3$). The same approach was used to determine the

equivalent concentration for AgNPs (shown here as an example for a reported concentration of $0.4\,\frac{\mu g}{mL}$ AgNP):

$$0.4\,\frac{\mu g}{mL} Ag(I)\ as\ AgNP * \frac{1\ g}{10^6\ \mu g} * \frac{1\ mol\ Ag(I)}{107.87\ g} = 3.71x10^{-9}\,\frac{mol\ Ag(I)}{mL} as\ AgNP \quad (2).$$

Since the silver salt ion control is at the same mass concentration as the AgNP ($0.4\,\frac{\mu g}{mL}$), the values are the same, which is expected given the assumption that the concentration represents the total silver atoms. This ion control assumes that the AgNPs completely dissolve, and thus delivers an ion concentration that exceeds the amount of Ag(I) ions that is realistically released from the AgNPs (*vide infra*). Example calculations using the alternative assumption are outlined in the Supplementary Information (SI). The percent of AgNP oxidation necessary to release Ag(I) ion concentrations equivalent to the ion controls used in the respective study was calculated as follows:

$$\%\ AgNP\ oxidation = \frac{\frac{mol}{mL} Ag(I)\ as\ AgNO_3}{\frac{mol}{mL} Ag(I)\ as\ AgNP} * 100 \quad (3).$$

To determine whether the necessary extent of oxidation is reasonable, the theoretical extent of oxidation was calculated under the assumption that only a single monolayer of the NP is available for oxidation. This assumption is empirically supported. For example, Sotiriou et al. found that the equilibrium Ag(I) ion concentration released from the particle into solution corresponds with the dissolution of one to two monolayers and is dependent on the particle size.[39] For particles greater than 8 nm, the mass fraction of released Ag(I) ions is equivalent to the mass of a single silver oxide monolayer, whereas dissolution of particles less than 5 nm in diameter corresponds to oxidization of two silver oxide surface layers.[39] An intermediate extent of oxidation, i.e., in between one to two silver oxide surface layers, appears for particles sizes

between 5-8 nm. We proceeded with the assumption that theoretical dissolution was equivalent to the oxidation of the outermost surface monolayer because 95.5% (64/67) of the AgNPs in the identified literature are greater than 5 nm (the three studies that include AgNPs less than 5 nm also included AgNPs with diameters greater than 5 nm). To estimate this monolayer, the percentage of surface atoms on a given AgNP was calculated as follows:

$$\%\, surface\ atoms = \%\, ionization = \frac{surface\ atoms}{total\ atoms}\ x\ 100 \quad (4).$$

The method used to calculate the volume (needed for determination of the total number of atoms) and surface area (necessary for determination of the total number of surface atoms) takes into account the size and shape of the AgNP used in a given study (equations in SI). For the determination of surface atoms, the percentages of different surface facets present on AgNPs of different shapes were also considered, as this factor can influence the number and packing of the atoms on the surface, as well as influence surface reactivity and the propensity to oxidize. Pseudo-spherical particles were the predominant particle shape studied in the identified literature subset and were approximated as cuboctahedrons, having eight (111) faces and six (100) faces, containing 63.4% and 36.6% of the surface atoms, respectively.[79] The monotonic relationship between nanoparticle size and the total number and percent of surface atoms is well established; the total number of surface atoms increases with increasing particle size and the percentage of surface atoms increases with decreasing particle size (see Figure 2 and Table 2 in reference 80).[80] Forty percent oxidation was determined as a conservative threshold for a scaled ion control based on (i) the available percent surface atoms (0.6-26% based on calculation) determined from the AgNP size and shape, and (ii) the potential for additional ionization due to known influences of experimental conditions (*vide supra*), which

cannot uniformly or robustly be considered in these calculations. As a result, ion controls that require > 40% of the AgNP to oxidize were considered overestimated. Following the example calculation above, the 1:1 ratio of Ag(I) ions as silver salt and AgNP would result in 100% of the AgNP needing to oxidize. This systematic review focuses solely on the results and conclusions presented in the subset of studies that included at least one scaled ion control. A schematic illustrating the approach to scaled ion control determination is presented in Figure 2.

Figure 2

These calculations enabled isolation of those studies that incorporated a scaled ion control that is representative of the total possible Ag(I) ion released from the AgNP studied. This narrowed the focus to conclusions drawn from these isolated studies with the goal of gaining clarity in the ion versus particle antimicrobial activity debate. Still, the calculations are not without limitations. First, the solvent environment (e.g., growth media),[28, 38, 42-44] the presence of bacteria, [27, 68] and surface chemistry (e.g., ligand identity, silver oxide formation),[24, 31, 33, 38, 45, 56, 57, 62, 67, 70, 81-83] among other factors, have all been shown to influence AgNP ionization (*vide supra*). Yet, the mechanisms remain unresolved and thus limit our ability to predict the influence of experimental conditions on Ag(I) ion release in the studies reviewed herein. Second, the calculations determine how much silver can theoretically ionize from the AgNPs indicating the bulk concentration of Ag(I) ion in solution, not the concentration of Ag(I) ion internalized by the bacteria (intracellular silver), which Ivask et al. reports as being a better indicator and comparison of antimicrobial activity.[24]

Once Ag(I) ion enters the bulk solution, there are many possible interactions that occur depending on the environment, including diverse binding events and equilibria that can inhibit

or drive ion release (*vide supra*). These interactions influence the fate of Ag(I) ions in a given experimental system and as a result, it is impossible to predict how many Ag(I) ions can and will enter the cell. Third, the calculations do not consider kinetics of ion release (i.e., the result represents the total possible dose of Ag(I) ion released from the AgNP). This omission is not to say that the studies reviewed do not consider kinetics, but rather that the kinetics of ion release are not included to determine whether a study incorporated a scaled ion control. Finally, these calculations are applicable to only particle diameters greater than 5 nm because the shapes are well-defined with easily calculable volume and surface area, have face-centered cubic (FCC) packing, and allow us to include a correction for the surface facets. As the particle diameter decreases to below 5 nm, the number of competing structural factors increases and the propensity for it to be referred to as a 'nanocluster' emerges. Different atom packing densities and arrangements become competitive and influence the shape and geometry of nanoclusters (e.g., the cuboctahedral geometry becomes icosahedral) so that they can no longer be easily approximated as spheres.[49, 84, 85] (Note: Only 3 studies included particles with diameters < 5 nm and those studies also included particles > 5 nm, so the calculations for the 5 nm NPs will not significantly influence the conclusions drawn in this study.)

3.3 Using pivot tables to identify AgNP properties and experimental variables that discriminate ion-only, particle-only, and combined ion-particle contributions to observed antimicrobial activity

The pivot table feature in Microsoft Excel (Ver. 2016, Microsoft Corporation, Redmond, WA) was used to identify discriminating factors influencing ion-only, particle-only, and combined ion-particle contributions to AgNP antimicrobial activity. Pivot tables are particularly

useful for summarizing and making sense of large, detailed data sets. Qualitative data (e.g., experimental method parameters, conclusion drawn from the study) and empirical data (e.g., particle size, zeta potential) was compiled for each study and organized into an Excel spreadsheet (Table S1), from which several analyses using pivot tables were conducted.

Conclusions from the identified subset of studies were categorized based on the study conclusions and accompanying data in support of an ion-only, particle-only, or combined ion-particle mechanism. Studies that attribute antimicrobial activity solely to Ag(I) ion release and concluded negligible particle-specific effects were categorized as 'ion-only'. Studies that identified and demonstrated the particle influence on antimicrobial activity – independent or in concert with Ag(I) ion release – were categorized as having a 'particle-only' or 'combined ion-particle' effect. For example, enhanced localized dissolution at the NP-cell interface or intracellular dissolution is enabled by the NP (as in the case of appending cationic capping ligands to guide targeted delivery[24]), yet the increased concentration of ions released in close proximity to the bacteria is often claimed to be responsible for the inactivation. The resulting enhanced bioavailability increases the antimicrobial impact over the equivalent concentration of bulk dissolved silver. The particle does not work independently from the ion, but the particle parameters can influence the magnitude of impact. In this paper, this mechanism is classified as 'combined ion-particle' since the ion and particle are both necessary to achieve this enhanced antimicrobial activity. However, because ion release occurs concurrently with particle-only effects, it is difficult to decouple 'particle-only effects' and 'combined ion-particle' effects, especially when multiple particle types and support for multiple mechanisms appear in one study. Some studies include support for multiple conclusions as a result of particle

manipulations and different methodology used to assess antimicrobial activity and so were categorized under multiple mechanisms.[24, 28] For example, in one study, there is evidence for 'combined ion-particle' effects when using one particle type, but 'particle-only' effects are supported when using another particle type.[24] The unknown mechanism through which the particle induces specific effects or enhances localized and/or intracellular dissolution adds to the complexity of decoupling these contributions, but further suggests the importance of the particle and the ability to shift mechanisms by manipulating particle parameters.

4. RESULTS and DISCUSSION

4.1 Identification and evaluation of studies implementing scaled ion controls

From the 59 studies identified as investigating ion-only, particle-only, and combined ion-particle contributions to bacterial cytotoxicity or antimicrobial activity, 30 studies (51%) included a scaled ion control as determined by the calculations presented above. Results of these calculations are compiled in Table S2. From these 30 studies, 22 studies (73%) measured Ag(I) ion release from the AgNP in the experimental system. The reported dissolution in these 22 studies strongly agreed with our calculated theoretical dissolution, which serves as further validation of the calculation method (see Table S2 and Figure S2 for demonstrated agreement of values). Additionally, this validation establishes the calculation method as a robust approach that can be used to inform scaled Ag(I) ion controls, especially if coupled with an empirical kinetic law of oxidation to estimate the kinetics of ion release, similar to those developed by Liu and Hurt for low AgNP concentrations[46] and Molleman and Hiemstra for pH-dependent and size-dependent ion release.[48] Of those 22 studies, the majority (74%) did not monitor the kinetics of Ag(I) ion release, but rather measured Ag(I) ion concentration at a single time point.

While monitoring of kinetic release was not a mandatory criterion for inclusion in our systematic review, it is an important aspect to consider given the wide variability of Ag(I) ion release than can occur and challenges associated with capturing kinetics of Ag(I) ion release (*vide supra*).

4.2 Support for ion-only, particle-only, and combined ion-particle contributions to AgNP antimicrobial activity

As stated earlier, the three possible contributions of the ion and particle to antimicrobial activity are supported by the literature and influenced by the physicochemical properties of the AgNP. Table 1 summarizes the influence of particle parameters (i.e., size, shape, and surface chemistry) on the ion and particle contributions outlined in each of the three possibilities.

Table 1

Isolating those studies that include a scaled ion control in their comparison of ion-only, particle-only, and combined ion-particle contributions to antimicrobial activity was intended to eliminate potentially confounding conclusions and to focus on those studies that offer robust discriminating conclusions. Of the 30 studies, 39% concluded that the observed antimicrobial activity results from an ion-only mechanism while 16% and 45% of studies concluded a particle-only and combined ion-particle mechanism, respectively (Figure 3).

Figure 3

These results indicate that the impact of the particle cannot be ignored and that there remains an opportunity to elucidate both underlying ion-independent antimicrobial mechanisms and mechanisms that act in concert with the Ag(I) ions as well as whether particle-specific parameters can be manipulated to influence these mechanisms. While the contribution

of Ag(I) ions to AgNP antimicrobial activity is largely indisputable, empirical support for ion-independent, particle-only effects suggests an opportunity to leverage particle property manipulation to further tune the mechanism and magnitude of impact. The precise mechanisms through which the particle induces antimicrobial activity are not resolved, yet particle size and surface chemistry are two critical factors that have been suggested and are further supported by the studies reviewed herein. [24, 31, 57, 65-67, 70, 71, 73, 86] The identified subset of literature included only one study[75] investigating the effect of particle shape, limiting our ability to draw any meaningful conclusions about the influence of shape.

4.2.1 Size-dependent mechanisms of AgNP antimicrobial activity

A size-dependent mechanism has been suggested to govern the antimicrobial activity of AgNPs with diameters ≤ 10 nm.[23, 24, 31, 33, 65, 66, 70-72] Of the studies that included AgNPs ≤ 10 nm, regardless of other sizes included, 71% concluded that particle-only or combined ion-particle effects dominate the antimicrobial activity (Figure 4B). (Note: This trend is not reflected in the '1-10 nm' size category of the main figure because studies including AgNPs ≤ 10 nm are split between the '1-10 nm' and 'range of sizes' categories.) The increased antimicrobial effect is suggested to result from more efficient contact with the bacteria[24, 66] and the increase of intracellular Ag(I) ions (determined by normalizing the EC_{50} values of the AgNPs to that of empirically determined intracellular Ag(I) ions[24, 66]). The mechanism through which intracellular silver is enhanced for particle diameters ≤ 10 nm remains unresolved, but is intriguing given that 10 nm has been identified as a threshold particle diameter below which theoretical ion release is increased due to enhanced surface reactivity (Ostwald-Freundlich equation),[45, 50, 51] meriting further research to resolve. One hypothesis is that at this length scale, particles are

able to enter the cell,[26, 28, 34-36, 82, 87] either through passive uptake (i.e., through porins in the cell wall) or as a result of altered membrane permeability. Yet, the reported size of bacterial porins are between 30-50 kDa, which correspond to 1-3 nm, making internalization via these pathways unlikely for some particle sizes.[23, 28] The combined ion-particle mechanism proposes that enhanced localized dissolution at the NP-cell interface or intracellular dissolution through a Trojan-horse-type mechanism is enabled through increased interaction between the NP and bacteria.[24, 33] Finally, as the particle diameter decreases to below 5 nm, different atom packing densities and arrangements may become dominant,[49, 84] influencing the shape and potentially enhancing the antimicrobial activity observed at this size range. Given that only 3 studies included particles < 5 nm,[27, 63, 88] this is a topic deserving further study.[51]

Ion-only and combined ion-particle mechanisms emerge as nearly equivalent contributions to antimicrobial activity for particle diameters greater than 10 nm with negligible particle-only contributions. Interestingly, 67% of the studies that conclude ion-only mechanisms of antimicrobial activity do not include ≤ 10 nm AgNPs (Figure 4B).[28, 62, 68, 69, 75, 89-91] Those studies that included a wide range of particle sizes (including AgNPs from two or more of the size categories included in Figure 4A) overwhelmingly (79%) concluded particle-only or combined ion-particle effects[24, 31, 57, 65-67, 70, 71, 73, 77] as opposed to studies that looked at a single size or narrow size range, of which 28% concluded particle-only or combined ion-particle effects.[23, 26, 33, 38, 72, 76, 86, 92] These results suggest that size is a factor inducing particle-specific effects that emerges only when multiple AgNPs are studied simultaneously. Including a range of sizes in a study will thus establish the relationship (e.g., monotonic, monotonic and linear, non-monotonic) between mechanism of antimicrobial activity and size as well as whether the

relationship is preserved across the full particle size spectrum. Adherence to or deviation from these relationships can provide support for either an ion-driven or, in this case, particle-specific or combined ion-particle mechanisms that would otherwise not be realized by studying a single particle size or narrow size range in isolation.

Figure 4

4.2.2 Surface chemistry-dependent mechanisms of AgNP antimicrobial activity

Typically, AgNPs are decorated with capping ligands, which are molecules that provide stability to the NPs, either through charge or steric repulsion. Ligands that contain functional groups such as amines ($–NH_2$) or thiols (–SH) typically have one chemical bond to the NP core, while other ligands such as trisodium citrate or poly(vinylpyrrolidone) have multiple interaction points. Ligands that have one bond to the NP surface have three key regions: the binding moiety (such as those mentioned above), the intramolecular region, and the solvent-facing moiety.[93] The solvent-facing moiety and the region of the capping ligand between the binding and solvent-facing moieties – the intramolecular region – can be used to introduce chemistries tailored for a given application (e.g., drug delivery, medical imaging, catalysis).[94-98] Each of these three regions, in isolation and/or in combination, may influence capping ligand density (number of capping ligands per unit surface area), interaction with the NP surface, and consequently Ag(I) ion release.[93] As a result, it is not surprising that the capping ligand is known to influence AgNP antimicrobial activity through ion release.[27, 33, 82] In addition to influencing chemical behavior (e.g., dissolution, passivation), surface chemistry has also been shown to impact physical behavior (e.g., aggregation, affinity for the bacterial cell).[45] Capping ligands specifically prevent aggregation by altering the electrostatic (Coulombic attraction between electric

charges) and/or steric (spatial interference causing hindrance) repulsion between NPs in dispersion.[49] The aggregation state of the NPs and their affinity for the bacterial cell have been shown to further influence AgNP antimicrobial activity.[24, 31, 57, 67, 70]

Due to the ability for surface charge to influence the aggregation state of AgNPs and the affinity for the bacterial cell, the subset of studies was organized around surface charge to determine potential influences on the conclusions drawn. The results reveal shifts in conclusions surrounding ion-only, particle-only, and combined ion-particle contributions to antimicrobial activity (Figure 5). We used zeta potential as the indicator of surface charge, which was included in 53% (16/30) of the studies in our identified subset. A large portion of studies (43%) that do not specify the ligand bound to the particle and do not include characterization of surface charge conclude the antimicrobial activity of AgNPs is governed by Ag(I) ions.[63, 64, 69, 82, 89, 91] Studies that used negatively charged capping ligands supported, in almost equal proportion, mechanisms that are governed by the released ions alone[27, 28, 62, 68, 75, 90] as well as synergistic effects of the ion and particle,[28, 33, 38, 57, 66, 73, 76] while only one study concluded a particle-only mechanism of activity.[86] Some of those studies supporting ion-only mechanisms, however, simultaneously varied capping ligand and particle size, which precludes isolation of a definitive conclusion surrounding the effect of the capping ligand. These studies also noted that surface chemistry-dependent antimicrobial activity did not always correlate with ionization, suggesting an additional factor (particle-driven) may be at play.[27, 62] Depending on interactions with media components, Ag-Cl and Ag-S could have formed a passivation layer on the AgNP surface that influences ion release.[42-45, 56] Only when both cationic NPs and anionic NPs are included in a study do particle-only or combined ion-particle mechanisms truly emerge.

Studies including multiple particle types (cationic NPs and anionic NPs) overwhelmingly (75%) supported a particle-only mechanism.[24, 31, 70] These results suggest that surface chemistry may be an influencing factor in inducing particle-specific effects, which is strongly supported when multiple ligand charges are compared. Since the particle is necessary to serve as the host for the capping ligand, the role of the particle is critical for introducing charge- (magnitude and type) and surface chemistry-induced antimicrobial activity.

Figure 5

Similar to NP size, the exact role of positively-charged capping ligands remains unresolved but it is speculated to govern a combination of indirect ROS formation, cell membrane disruption, and/or bioavailable intracellular silver. AgNPs with positive charges due to positively-charged capping ligands (hereafter referred to as cationic AgNPs) demonstrate enhanced antimicrobial activity compared to their neutral and negative counterparts, which is attributed to their electrostatic attraction and subsequent adherence to the negatively-charged bacterial cell surface.[24, 31, 70] These observed effects are not attributed to the Ag(I) ion, since less bulk dissolution occurred with the cationic AgNPs studied than for other particle types (e.g., AgNPs with negative charges) and is present in smaller amounts than the silver salt control.[24, 70] However, the affinity of the cationic AgNP for the negatively-charged cell allows for enhanced localized release of Ag(I) ions, internalization of the particle and/or ions, and ROS production at the cell membrane.[24] Furthermore, cationic AgNPs induce physical damage to the cell membrane (i.e., pitting observed by TEM)[31] that disrupts ion efflux systems, which in turn hinders the pumping efficiency of Ag(I) ions out of the cell, thus increasing intracellular ROS generation compared to exposure to the same amount of Ag(I) ions from the equivalent silver

salt control or other particle types.[77] Additionally, cationic AgNPs increase the amount of intracellular Ag(I) ions that deactivate antioxidative enzymes, potentially allowing for the buildup of ROS to occur.[4] The pathway through which the cell mitigates stress caused by cell membrane-associated ROS from cationic AgNPs was found to be similar to the pathway demonstrated by cationic polystyrene NPs, suggesting a particle-type specific response is elicited for cationic particles independent of the particle core composition.[24]

In addition to the pH-dependent charge of the terminal moiety of the ligand, the molecular weight, ligand density (number of capping ligands per unit surface area), and chemical composition of the capping ligand may also influence antimicrobial activity and the mechanism through which it occurs. While one study in the identified subset[33] supports the influence of all three regions of the capping ligand on bulk and intracellular dissolution and found antimicrobial activity to increase with this dissolution, the remaining studies lacked critical information (e.g., the absence of comprehensive capping ligand characterization, particularly those corresponding to polymer- and protein-based capping ligands) to be able to organize in a pivot table around binding moiety, intramolecular region, and terminal group. Still, the following discussion includes potential influences of the multiple capping ligand components.

First, the NP-binding moiety influences dissolution; atoms with weaker binding affinities (e.g., oxygen versus sulfur) enable adsorption exchange with ambient oxygen and allow increased Ag(I) ion release to occur.[33] Second, the molecular weight of the intramolecular region influences the bulk density of organic matter surrounding the AgNP core, which can "shield" particle and ion effects (e.g., influence the release of Ag(I) ions to the bulk solution).[33,

[99] Third, terminal groups of the capping ligands have different affinities to interact with Ag(I) ions, so the amount of Ag(I) ions remaining on the particle surface and being leached into solution can be different.[33] For example, it may be possible that the positively-charged Ag(I) ions electrostatically interact with capping ligands having negatively-charged terminal groups, limiting the amount of bioavailable Ag(I) ions. Finally, ligand density influences the interaction between the NP surface and surrounding environment (e.g., dissolved O_2), and consequently, should influence Ag(I) ion release.[93] Ion release is hypothesized to scale inversely with initial ligand density (i.e., more ion release occurs with a lower initial ligand density because more of the AgNP surface is exposed for oxidation). No studies have directly measured how the initial ligand density affects ion release, but a recent study measured *changes* in ligand density after AgNPs were incubated in bacteria growth media, where the hypothesis was that AgNPs with a larger decrease in ligand density would also have greater Ag(I) ion release. However, no correlation was found between the change in ligand density and the degree of Ag(I) ion release.[38] This complex relationship between ligand density and ion release illustrates a need for further research aimed at establishing ion release profiles of multiple ligand chemistries and densities and resolving the contribution of surface chemistry to antimicrobial activity. In doing so, opportunities to tune ion-driven antimicrobial activity through controlled manipulation of the AgNP surface will be revealed.

4.2.3 Effect of aggregation on ion-only, particle-only, and combined ion-particle contributions to antimicrobial activity

Aggregation of AgNPs can occur in aqueous environments due to an assortment of factors influencing surface reactivity (e.g., particle size, shape, surface coating, crystallinity, and

exposure media),[49] and can suppress mechanisms activated by unique nanoscale properties of a given nanomaterial. Despite the prominence of aggregation phenomena, many studies do not characterize or else do not explicitly report aggregation behavior. Additionally, a poorly recognized critical factor in managing AgNP aggregation is the method of AgNP addition to the exposure media. For example, diluting AgNPs in water before introducing them to growth media can help retain the size and stability of the particle.[69]

In the identified subset of literature, some studies consider aggregation early in the experimental design as demonstrated through the choice of low ionic strength growth media or in the ligand selection to enhance particle stability,[26, 82, 92] but may not actually monitor aggregation under experimental conditions. Interestingly, the inclusion or absence as well as the method used to monitor aggregation impacts the conclusions drawn in these studies (Figure 6).

Figure 6

When aggregation is not characterized, no conclusion regarding the mechanisms of antimicrobial activity can be discerned.[26-28, 64, 67, 71, 75, 91, 92] The majority of these studies noticeably did not specify the ligand bound to the particle and did not include characterization of surface charge, providing no indication about the stability of the AgNPs, thus increasing the potential for elimination of particle-specific effects. Of those studies that characterize aggregation, dynamic light scattering (DLS) is the most common method used to monitor the change in hydrodynamic diameter and/or zeta potential of the AgNP suspension. The majority of studies (77%) employing DLS conclude particle-only or combined ion-particle effects,[24, 31, 33, 57, 66, 70, 73, 76, 86] and whether aggregation was monitored throughout the duration of the

experiment or pre- and post-experiment had no effect on the conclusion being drawn. When imaging techniques (e.g., TEM, scanning electron microscopy (SEM)) were employed, there was a slight predominance of ion-only conclusions. N. B. There are inherent challenges to characterizing particle aggregation state via electron microscopy, because samples are prepared and subsequently imaged outside of their native solution, and the removal and subsequent sample preparation (most notably, sample drying) influences particle-particle interactions and may not reflect the native dispersed state. Finally, when multiple methods are employed and considered together to determine the influence of aggregation on the antimicrobial activity of AgNPs, the majority of studies conclude particle-only or combined ion-particle effects. Taken together, the analysis suggests that aggregation is an important factor influencing ion-only, particle-only, and combined ion-particle contributions to antimicrobial activity, and as such, is critically important to monitor and consider when drawing conclusions within a given experimental system.

4.3 Experimental methods used to distinguish ion-only, particle-only, and combined ion-particle mechanisms influences the conclusion drawn

While the scaling of the ion control is one experimental factor we used to isolate the subset of literature and eliminate confounding effects, we identified the method used to decouple ion and particle contributions as an additional factor influencing the conclusions with respect to those contributions. Other experimental factors that were considered and found to have a less significant independent influence on conclusions drawn (i.e., resulted in a nearly equal number of studies concluding ion-only, particle-only, and combined ion-particle) include the AgNP dose, purification and washing procedure, method of dissolution monitoring, time of

dissolution monitoring, and the gram stain of the organism used (Figures S3-S7). There are numerous methods available to study interactions and the impact of ENMs on bacteria, which vary based on the type and resolution of information obtained. Within the subset of literature reviewed herein, the methods used to distinguish ion and particle contributions are grouped into the following categories: (i) Ag(I) ion release is quantified (either in the bulk solution or intracellularly) and compared to AgNP exposure scenarios,[23, 24, 57, 63, 64, 66, 68, 72, 73, 75, 77] (ii) the experimental system is designed to create a particle-only exposure (e.g., anaerobic environments eliminate oxidation of Ag(0) to Ag(I)),[27, 28, 33, 62, 82] and (iii) molecular or fluorescent indicators are used to resolve specific mechanisms of interaction. [23, 24, 26, 31, 33, 62, 63, 65, 68, 71, 72, 76, 77, 82, 90-92] Figure 7A shows the breakdown of conclusions drawn using these three general methods, each discussed in detail below.

Figure 7

4.3.1 Quantifying Ag(I) ion released in AgNP exposure conditions provide support for particle-only and combined ion-particle contributions to antimicrobial activity

One approach to elucidating particle contributions of AgNP antimicrobial activity is to account for or subtract the effect of the ion from the effect of the combined ion-particle system. This approach requires isolation and quantification of Ag(I) ion from complex aqueous systems. The two prominent methods to quantify the concentration of silver released from the AgNP as Ag(I) ion include (i) using a Ag(I) ion-selective electrode or UV-vis, or (ii) isolating dissolved Ag(I) ion followed by quantification typically by ICP-MS or AES or GF-AAS. In the identified literature subset, average bulk ion release ranges from < 1% to 16% of the AgNP. The majority of studies across varying reported percentages of bulk ionization occurring in the AgNP

system conclude particle-only or combined ion-particle effects, suggesting that the mechanism of antimicrobial activity is not due to the particle releasing more Ag(I) ions into the bulk solution (Figure 8). For example, when < 2% or between 2-4% ion release occurs, particle-specific effects dominate, which is expected when the ion fraction is low.[100] However, particle-specific effects continue to dominate at higher ion fractions (8-10%).

Figure 8

With the bulk dissolved Ag(I) ion concentration quantified, a specific approach to isolating ion-only, particle-only, and combined ion-particle contributions is to normalize the measured endpoints (e.g., the minimum inhibitory concentration (MIC) or the concentration that affects 50% of the test population (EC_{50})) or dose-response curves obtained under AgNP exposure conditions to the quantified concentration of Ag(I) ion to then compare the normalized impact with the corresponding silver salt control.[23, 24, 57, 63, 64, 66, 68, 72, 73, 75, 77] The results are interpreted as particle-specific or combined ion-particle effects when the magnitude of impact of the AgNP exposure condition is greater than that of the equivalent released Ag(I) ion concentration dosed in as a silver salt. When the measured endpoints or growth curves are similar or when the equivalent released Ag(I) ion imparts greater impact on the bacteria growth curve, the results are interpreted as being governed by the ion. Studies using this normalization approach predominantly (67%) conclude particle-specific or combined ion-particle effects govern the observed impact of AgNPs (Figure 7B).[23, 24, 57, 66, 72, 73, 77]

Still, definitively isolating different forms of silver in these complex systems remains an ongoing challenge in the field. The described current best-practice techniques capture free Ag(I) ions present in solution; they do not measure intracellular silver content or resolve the fraction

of Ag bound to the surrounding environment (i.e., ligands, media constituents, and components of the bacteria cell), as some Ag-complexes will partition/be captured in the supernatant and others will be pelleted, depending on the size of the complex and the separation technique being used. Additionally, studies that measure ionization at a single time point (oftentimes at the culmination of the experiment) exclude the kinetics of ion release. As mentioned above, oxidation of the AgNP surface is a dynamic process and is highly dependent on the surrounding aqueous chemistry, the specifics of which are not comprehensively resolved. As a result of our inability to capture the complexity of the system, we are currently unable to mimic the kinetics of ion release from AgNPs in ion-only exposures, limiting our ability to further distinguish ion-only, particle-only, and combined ion-particle mechanisms of antimicrobial activity.

In an effort to circumvent these limitations, an alternative method is employed in which intracellular silver is measured using a genetically engineered Ag(I)-biosensor bacteria, *Escherichia Coli* (*E. coli*) MC1061 (pSLcueR/pDNPcopAlux).[24, 66, 75] This recombinant bacterial strain harbors two plasmids from the copper resistance system essential for sensor function: (i) pSLcueR, which contains genes for the regulatory protein *cueR,* and (ii) pDNPcopAlux, which contains the *lux*-cassette (a group of bioluminescence encoding genes) fused to the promoter *pCopA.*[101] The protein *cueR* resides in the cytosol and tightly binds both cytoplasmic copper ions (Cu(I)) and Ag(I) ions due to their similar binding affinities, ionic radii, and charge densities.[26] Upon binding, *cueR* activates expression of *copA*, a Cu(I)/Ag(I)-translocating P-type ATPase involved in copper and silver efflux, and the *lux*-cassette required for the production of bioluminescence.[26, 101] This method measures strictly intracellular Ag(I) ions because it is the Ag(I) ion binding to the cysteine residues on *cueR* that is essential to induce bioluminescence.

The more binding events, the stronger the bioluminescent signal (in relative light units), thus serving as an indicator of intracellular Ag(I) ion concentration.[66] Conclusions from studies employing this methodological approach predominantly (67%) support particle-only or combined ion-particle effects.[24, 66]

4.3.2 Methodological approaches that create particle-only exposures support an ion-only mechanism of AgNP antimicrobial activity

Subtracting the effects of the released Ag(I) ion is a time and resource intensive endeavor, and quantifying intracellular silver captures only a portion of silver that impact the bacteria (albeit, the portion having a predominant adverse impact). An alternative approach involves establishing an exposure environment that eliminates free Ag(I) ions, allowing for deduction of particle-only effects. The two leading approaches to establish this 'particle-only' exposure condition involve (i) anaerobic environments, or (ii) the addition of a compound that sequesters free Ag(I) ions.

4.3.2.1 Anaerobic conditions

Formation of Ag(I) ions from the AgNP surface is an oxidation process. In anaerobic environments, oxygen is removed from the system, thus eliminating oxidative Ag(I) ion release. To eliminate the potential for microbial differences to confound results acquired in anaerobic studies, it is important to use a bacterial strain that grows under both aerobic and anaerobic conditions and exhibits equal susceptibility to Ag(I) ions in both conditions.[27, 28] The AgNP concentrations that elicit complete inhibition vary among anaerobic studies, ranging from above 195 mg/L[27] to 5 mg/L[28, 102] AgNP (compared to 75 mg/L[27] to 1 mg/L[28, 102] AgNP under aerobic conditions). While this approach is aimed at eliminating the Ag(I) ions and marginal

concentrations are observed after multiple days (e.g., less than 1 μg/L Ag(I)[27]), other studies report detectable Ag(I) ions under anaerobic conditions, reaching the same level as in the comparative aerobic conditions.[102] Evidence for Ag(I) ion release in anaerobic conditions is one potential explanation for the significant discrepancy in AgNP concentrations eliciting complete inhibition in the above-mentioned studies. Additionally, Ag(I) ion release can occur through non-oxidative means. AgNPs can release chemisorbed Ag(I) ions, which result from the partially oxidized AgNP surface even in the absence of an oxidizer.[45, 102, 103] The presence of chemisorbed and "free" Ag(I) ions will vary between studies based on (i) the exposure conditions (i.e., in the presence of bacteria whose metabolic state can change the pH to influence dissolution and extracellular polymeric substances released from bacteria that can sequester Ag(I) ions),[27] and (ii) the AgNP synthesis and purification process.[31, 39, 45, 102] For example, when mild reducing agents (e.g., sodium citrate) are used during the synthesis, some of the silver salt may not undergo reduction and thus remain as free ions in solution or bound to the AgNP surface.[45] As such, the absence of free Ag(I) ions in solution does not preclude the presence of chemisorbed Ag(I) ions, which could be transported to the cell by the AgNP. Additionally, if particle internalization occurs and the cytosolic pH is lower than that of the growth medium, intracellular release may occur in anaerobic bacteria. Since nitrate compounds are used as electron acceptors in anaerobic bacteria, reactive nitrogen species (e.g., nitrogen dioxide, nitrous oxide) may also form intracellularly,[104] acting as an oxidizing agent to induce Ag(I) ion release. To test this combined ion-particle hypothesis, intracellular silver could be measured using an anaerobic Ag-biosensor.

Nonetheless, when designed to avoid these potential confounding experimental factors, anaerobic exposure environments are good model systems to probe ion and particle contributions independently. Current anaerobic studies (2 reviewed herein) provide convincing support for Ag(I) ion-only mechanisms but do not conclusively rule out particle-only contributions since particle parameters (e.g., varying size and surface chemistry) have not been comprehensively varied under anaerobic conditions. An inability to demonstrate particle-only effects does not prove an ion-only mechanism. Studying a comprehensive AgNP suite that systemically varies shape, size, and surface chemistry under anaerobic conditions is necessary to rule out particle-only effects. Finally, these studies have considered a single bacterial strain and so multiple bacterial strains, having different defense systems for ions and particles, should be evaluated under anaerobic conditions.

4.3.2.2 Sequestering Ag(I) ions

Another approach to eliminate the effect of Ag(I) ions without precluding their formation is to introduce a compound that sequesters free Ag(I) ions to the system. This approach reduces the bioavailability of the Ag(I) ion, thereby minimizing interaction with the bacteria. Commonly used sequestering compounds include cysteine, chloride, sulfide, thiosulfate, phosphate, and glutathione reductase,[28, 33, 62, 82] which demonstrate efficient binding with Ag(I) ion in both AgNP and silver salt exposure conditions. While half of the studies employing this approach conclude an ion-only mechanism of antimicrobial activity (Figure 7B),[62, 82] there is also evidence supporting the combined ion-particle possibility and specifically, particle-mediated intracellular ionization.[28, 33] One limitation of this approach is that the sequestration compounds also have a strong affinity for the AgNP and upon binding, could

influence the NP surface chemistry and hinder the particle-only and combined ion-particle effects.[32] Furthermore, the complexed silver may still exhibit bioavailability and induce antimicrobial activity that may be different than the effect of free Ag(I) ions.[105] Despite the fact that this approach successfully establishes an ion-free system to aid in decoupling ion and particle contributions, it alters the system in a way that does not allow for a direct comparison.

4.3.3 Methods used to elucidate specific mechanisms of AgNP-bacteria interactions support ion-only, particle-only, and combined ion-particle contributions to antimicrobial activity

While linking biological endpoints to the presence of Ag(I) ions and the AgNP aids in determining ion-only, particle-only, and combined ion-particle contributions, there are also techniques that adopt the same systematic approach while simultaneously providing information on specific pathways of inactivation. The identified methodologies include using multiple bacterial strains with distinguishing phenotypes, employing microscopy techniques, and using molecular-level techniques (e.g., gene expression and gene-deletion strains).

A suite of gram-negative and gram-positive bacteria can be used to study how differences in the cell wall – the first point of contact in the AgNP-bacteria interaction – influence the response to Ag(I) ions and AgNPs. Gram-negative bacteria have a thin peptidoglycan cell wall and an outer cell membrane, while gram-positive bacteria have a thicker peptidoglycan layer and no outer cell membrane.[76] These differences in architecture introduce different susceptibilities and defense systems to AgNPs and Ag(I) ions in a way that reveals different mechanisms (Figure 7B). It is critical, however, that both bacteria strains are able to grow in the same exposure media to eliminate confounding effects associated with differences in interactions with the surrounding media. Thirteen percent (4/30) of the studies reviewed

herein include both gram-negative and gram-positive bacteria,[72, 76, 82, 90] half of which showed that Ag(I) ions and AgNPs induced different magnitudes of antimicrobial activity suggesting different modes of action of the two forms of silver.[72, 76] One study shows AgNPs having greater antimicrobial activity in gram-positive bacteria compared to gram-negative bacteria while the Ag(I) ions induced approximately equivalent antimicrobial activity in both.[76] On the contrary, the thick peptidoglycan wall of the gram-positive bacteria is proposed to obstruct ions,[72, 90] rendering the action of Ag(I) ions alone less effective in gram-positive bacteria and suggesting that particle attachment to the cell surface contributes to the antimicrobial activity in a particle-only or combined ion-particle manner. However, different AgNP surface coatings can prevent this attachment, which explains why an earlier study reported AgNPs as having less antimicrobial activity in gram-positive bacteria.[72] Using multiple bacterial strains and particle types can differentially affect the antimicrobial activity of Ag(I) ions and AgNPs. This outcome underscores the importance of implementing well-defined and well-controlled materials and biological systems to comprehensively study that differential impact and isolate the contributions of the ion and particle. Using gram positive and negative bacteria in combination with the anaerobic and/or sequestration approaches would further resolve in the influence of the bacterial cell wall on the susceptibility and dominant mechanisms of antimicrobial impact.

Capturing visual evidence of Ag(I) ion and AgNP interactions with bacterial cells as well as the resulting morphological changes can aid in determining the contributions of each to the observed antimicrobial activity. Common microscopy techniques used include transmission TEM, SEM, atomic force microscopy (AFM), and phase contrast microscopy.[24, 26, 31, 33, 62, 63, 68, 71, 76, 82, 91] In the studies reviewed, nearly 40% relied on a microscopy technique to image bacteria-

NP interactions and cell morphology,[24, 26, 31, 33, 62, 63, 68, 71, 76, 82, 91] of which 42% concluded an ion-only mechanism,[62, 63, 68, 82, 91] 17% concluded particle-only effects,[24, 31] and 42% concluded a combined ion-particle mechanism[24, 26, 33, 71, 76] (Figure 7B). Direct AgNP contact with and fusion to the membrane, shrinkage of cell size to reduce its surface area available for interaction, particle entry into the cytosol, resulting membrane damage (e.g., pitting, rupture), and subsequent leakage of intracellular components occurs in AgNP exposures exclusively, suggesting that these physical effects are the result of the AgNP itself and are not dependent on Ag(I) ions.[24, 31, 71, 76] Ag(I) ions, on the other hand, have also been visualized in cells as large deposits of reduced Ag(I) (silver nanoparticles) that destroy the cell[82, 91] but may also induce another bacterial response, often culminating in the clumping of DNA in the cell center.[106] While internalization and morphological changes may be visualized and used to isolate physical contributions of the particle from the chemical contributions of the Ag(I) ions, the mechanism by which each enters the cell remains unknown. The observed presence of 'particles' inside the cell could instead be Ag(I) ion agglomerates or formed by metabolite reduction intracellularly.[45, 82] Finally, TEM imaging on its own is not a convincing technique for visualization of particle uptake by cells due to the potential for a variety of misleading artifacts.[26]

Using molecular-level techniques (e.g., transcriptomics, gene deletion strains) to distinguish ion-only, particle-only, and combined ion-particle contributions are valuable due to the ability to concurrently elucidate the underlying mechanism(s) of antimicrobial activity. Given that these molecular approaches target specific mechanisms, the results of a given study might be limited in scope due to the focus on isolating a particular ion or particle interaction or impact. Still, the resulting conclusions from the 20% (6/30) of studies using molecular-level

techniques are overwhelmingly (86%) in support of particle-only or combined ion-particle effects (Figure 7B).[23, 24, 26, 71, 92] The compiled findings from the 6 studies provides valuable mechanistic insight. For example, genome-wide approaches show differential gene regulation under AgNP and Ag(I) ion exposures and are used to highlight particle-only[23, 24, 71] and particle-type specific mechanisms (e.g., cationic AgNPs).[24] Specifically, particles induce antimicrobial activity by affecting cell surface activity through disruption of outer membrane liposaccharide formation.[24, 92] These particle-only effects differ from the action caused by Ag(I) ions alone, which include disruption of copper homeostasis, inducement of additional redox stress responses, and regulation of outer membrane porin proteins.[23, 92] However, the interpretation of genetic responses can lead to different conclusions about ion and particle contributions. For example, McQuillan and Shaw credit the differential genetic response between AgNPs and Ag(I) ions to the delivery mode of Ag(I) ions to the cell that affects the magnitude, location, and kinetics of Ag(I) ion release rather than to particle-only effects, so this combination is considered a combined ion-particle mechanism.[92] Despite the challenges associated with interpretation, molecular-level techniques provide resolution of the mechanism of antimicrobial activity and can highlight particle-specific stress responses that occur at small time scales,[26, 92] with longer time scales deserving attention to study how resistance can build up over time and through multiple generations.[15]

5. CONCLUSIONS AND FUTURE OUTLOOK

Results from the systematic review on the AgNP antimicrobial activity literature provide supporting evidence for particle-specific effects that act both in concert with as well as independently from the Ag(I) ion. The discriminating pivot table analysis suggests the potential

to create more effective, Ag-enabled antimicrobials using critical particle properties – size, shape, and surface chemistry. The following are suggestions for ongoing research to this end.

Further research is necessary to elucidate the contributions of each NP property (both their independent and combined effects) and would be most productively pursued by using experimental conditions that have successfully demonstrated the ability to distinguish ion and particle contributions (e.g., anaerobic environments). Given that NP morphology is known to influence surface reactivity and physical interactions with the bacteria membrane, particle shape is a topic deserving investigation that could provide insightful contributions to resolving the particle-ion mechanisms. Additional resolution of the influence of environmental constituents on surface chemistry of AgNPs is needed since Ag(I) ion release and bacterial interactions are both surface phenomena and these constituents can influence ligand chemistry as well as promote or inhibit oxidation of the NP surface. As a result, more comprehensive characterization of surface chemistry will be necessary for future studies, including monitoring of changes in surface chemistry that result from exposure to the experimental system. Finally, complete resolution of the ion versus particle debate requires development of a robust approach to isolating and quantifying released Ag(I) ions in AgNP exposure conditions, specifically Ag(I) ions bound to media constituents or bacterial components. This development will allow for an accurate representation of the AgNP to be modeled with Ag(I) ion-only exposures by delivering a series of doses that mimic the total rate of AgNP dissolution – the preferred and suggested best practice for an ion control. These recommendations for minimal characterization and reporting guidelines in future studies are summarized in Table 2.

Table 2

While this systematic review is limited to bacteria, the importance of elucidating the mechanism of action and contribution of the ion and particle is ubiquitous across/traverses other types of organisms (e.g., algae, fungi, viruses, mammalian cells). In fact, it is critical from a human and environmental health standpoint that AgNPs used as antimicrobial agents be designed to maximize efficacy while minimizing harm to organisms higher up on the trophic level. Elucidating the mechanism of action and contribution of ion and particle across trophic levels is important and is likely to shift due to organism-specific responses. For example, endocytosis mechanisms of eukaryotic cells support potential particle-driven and combined ion-particle mechanisms of action. Our conclusions are not directly transferrable across different model organisms due to such phenotypic differences, yet the abovementioned best practices of AgNP characterization and use of scaled ion controls do translate across trophic levels and are critical to decoupling ion and particle contributions to the mechanism and magnitude of impact.

In addition to resolving the independent and synergistic contributions of the ion and, such research activities will inform the tuning of particle design specific to the intended application. Given the rising demand for effective antimicrobials, particularly in the wake of the global antimicrobial resistance crisis, there is a need to inform the use of AgNPs as antimicrobials. More generally, the mechanistic knowledge gained can be used to inform design of other effective nano- and non-nano alternatives, particularly the delivery of multiple mechanisms of inactivation.

6. CONFLICTS OF INTEREST

There are no conflicts to declare.

7. ACKNOWLEDGEMENTS

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9. FIGURES AND TABLES

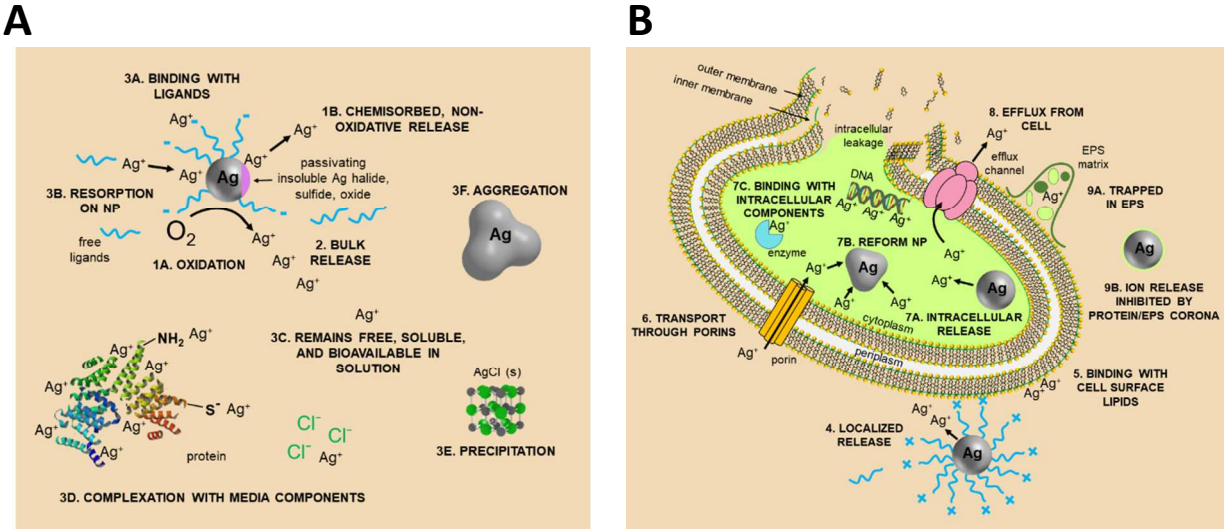


Figure 1. Potential Ag(I) ion release pathways, binding, and partitioning events within the AgNP exposure condition. (A) State of Ag in the media, external to the bacterial cell. (B) Interaction of silver with the cell and state of Ag inside the cell. Note: figure is not to scale as the particle is enlarged to demonstrate effect.

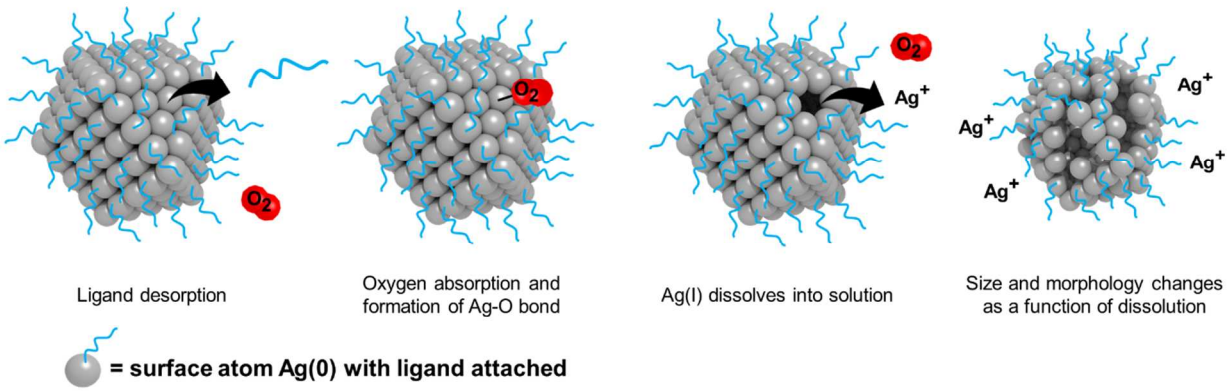
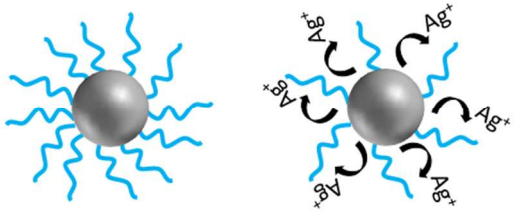
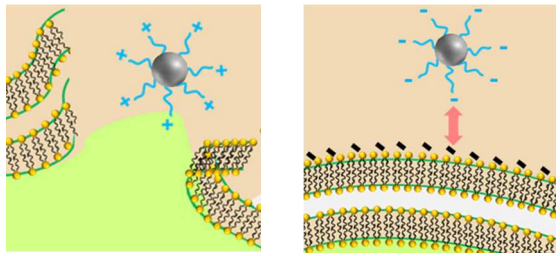
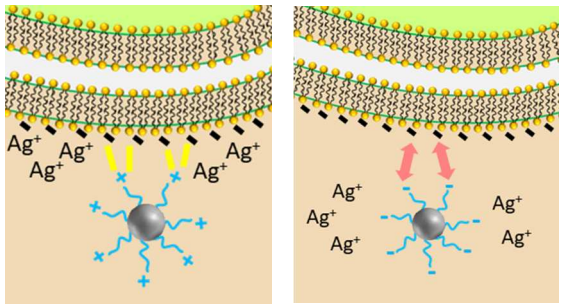
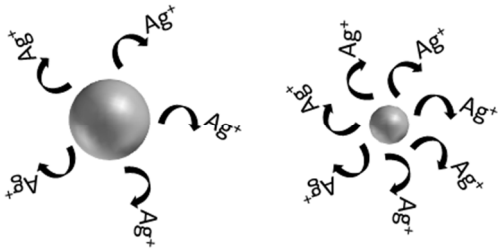
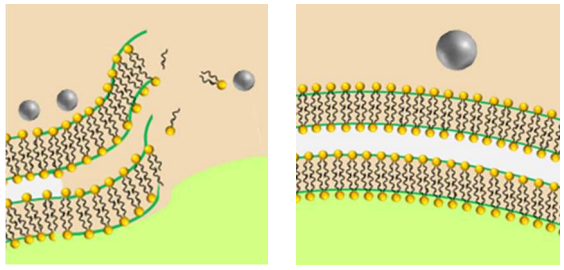
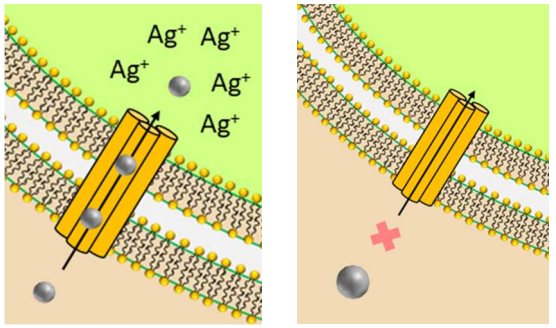
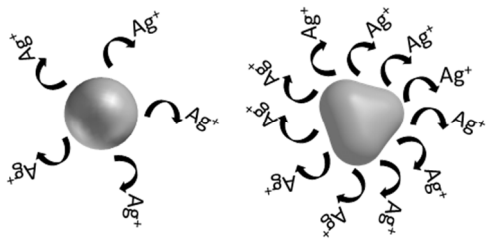
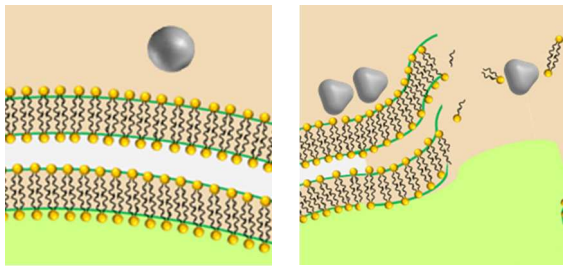
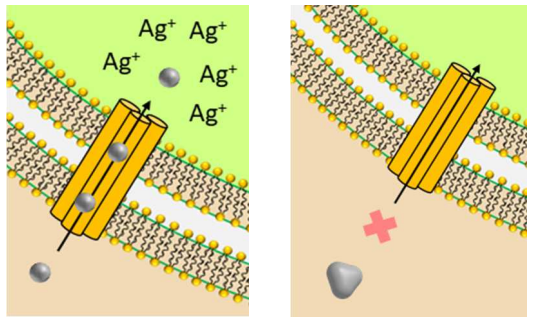


Figure 2. While silver dissolution is a complex and dynamic process, this schematic illustrates a step in the theoretical dissolution of a given AgNP monolayer. The depicted process serves as the underlying assumption for establishing the threshold for a scaled ion control. The atom-by-atom surface dissolution of AgNPs initiated by (i) ligand desorption, (ii) oxygen sorption and formation of the Ag-O complex that begins oxidation of Ag(0) to Ag(I), (iii) Ag(I) dissolves into the continuous phase, and (iv) size and morphology of the particle changes as a function of dissolution.

Table 1. Summary of ion-only, particle-only, and combined ion-particle hypotheses for AgNP antimicrobial activity as influenced by different particle parameters.

Particle parameter	Ion-only mechanism	Particle-only mechanism	Combined ion-particle mechanism
Surface chemistry	Ligand density and ligand chemistry influences ion release into bulk solution 	Charge influences attraction and repulsion to bacteria cell, further influencing contact with cell and damage through a physical mechanism 	Charge influences attraction and repulsion to bacteria cell, further influencing localized ion release 
Size	Surface reactivity due to size influences ion release into bulk solution 	Size influences passive uptake and subsequently contact with cell and damage through a physical mechanism 	Size influences passive uptake and subsequently intracellular ion release 

Shape	Surface reactivity due to shape influences ion release into bulk solution 	Shape influences contact with cell and damage through physical mechanism 	Shape influences localized and/or intracellular ion release 

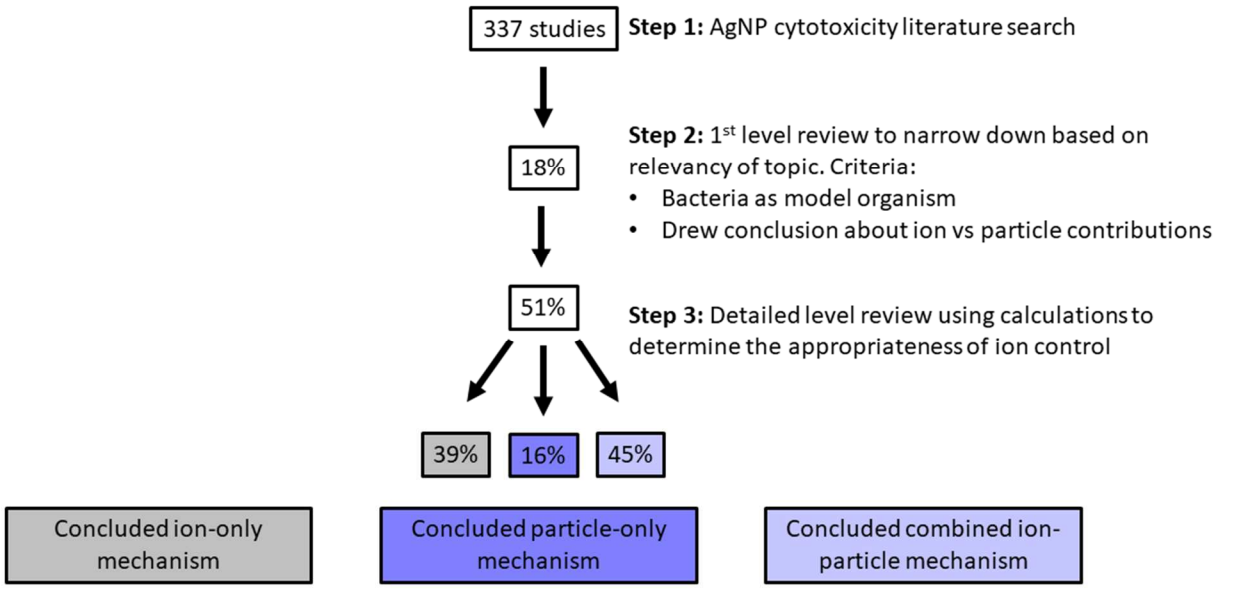


Figure 3. Scheme of the literature review process. The percent in the box is equal to the percent of studies fulfilling the requirement for that particular step. Only 30 studies (51%) included a scaled ion control. Of those studies, 39% concluded an ion-only mechanism (stating that the particle does not play a role) while 16% and 45% concluded a particle-only or combined ion-particle mechanism (stating that the particle plays a role).

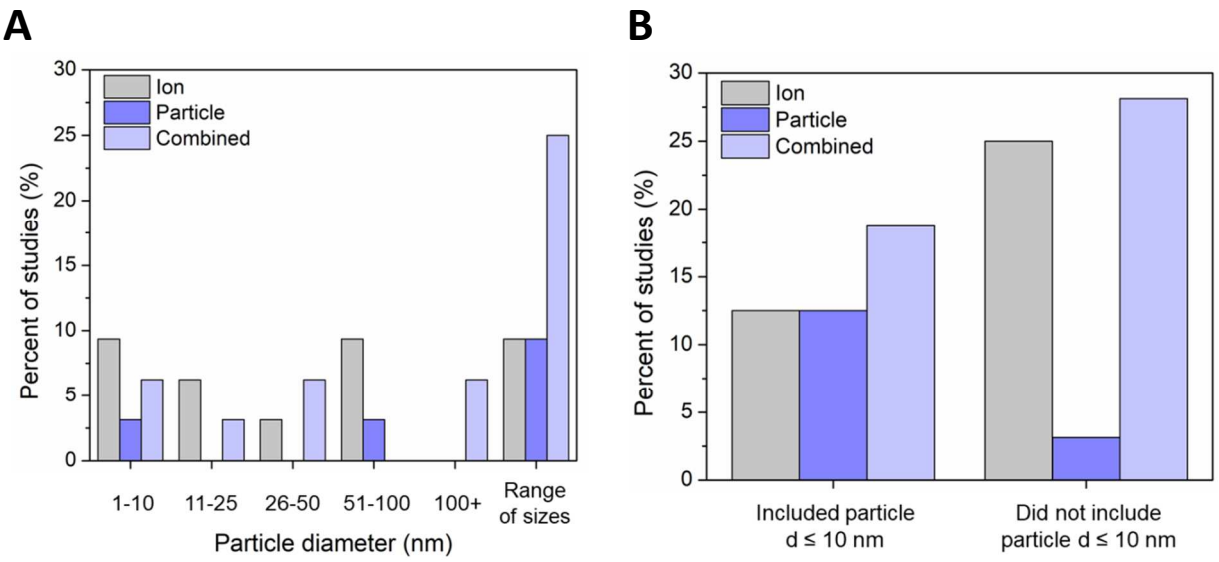


Figure 4. (A) Ion-only, particle-only, and combined ion-particle conclusions drawn from studies that include AgNPs only within the size range of 1-10 nm,[23, 33, 63, 64, 72, 82] 11-25 nm,[38, 62, 68] 26-50 nm,[28, 76] 51-100 nm,[75, 86, 90, 91] greater than 100 nm,[26, 92] and AgNPs from more than one size range.[24, 27, 31, 57, 65-67, 69-71, 73, 77, 89] (B) Ion-only, particle-only, and combined ion-particle conclusions drawn from studies that include[23, 24, 27, 31, 33, 63-66, 70-72, 82] and exclude[26, 28, 38, 57, 62, 67-69, 73, 75-77, 86, 89-92] AgNPs with d ≤ 10 nm. Size is an influencing factor inducing particle-specific effects, in which the role of the particle emerges only when multiple particle sizes from different size categories are included. The role of the particle also emerges when particle sizes ≤ 10 nm are included in the study. Note: This trend is not reflected in the '1-10 nm' size category of the main figure because studies including AgNPs ≤ 10 nm are split between the '1-10 nm' and 'range of sizes' categories.

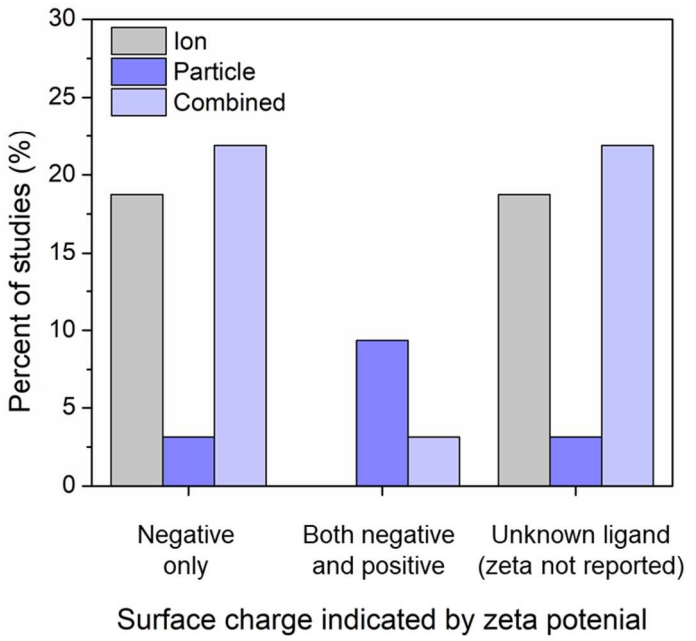


Figure 5. Ion-only, particle-only, and combined ion-particle conclusions drawn from studies that include AgNPs with a single capping ligand type inducing a negative surface charge,[27, 28, 33, 38, 57, 62, 66, 68, 73, 75, 76, 86, 90] two capping ligand types to compare a negative and positive terminal group,[24, 31, 70] and unknown capping ligands.[23, 26, 63-65, 67, 69, 71, 72, 77, 82, 89, 91, 92] Surface chemistry is an influencing factor in inducing particle-specific effects, in which the role of the particle emerges only when both negatively-charged and positively-charged particles are compared. Note: No studies included a positively-charged or neutral capping ligand only.

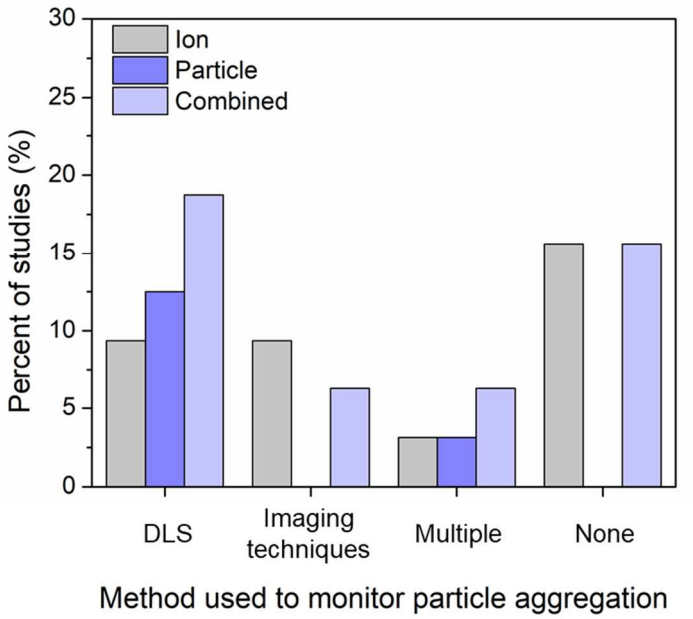


Figure 6. Ion-only, particle-only, and combined ion-particle conclusions drawn from studies that monitor particle aggregation using hydrodynamic diameter and/or zeta potential from dynamic light scattering (DLS),[24, 31, 33, 57, 66, 68-70, 73, 76, 86, 90] size and morphology from imaging techniques (e.g., TEM, SEM),[23, 63, 77, 82, 89] more than one technique (e.g., DLS, TEM, and UV-vis),[38, 62, 65, 72] or none at all.[26-28, 64, 67, 71, 75, 91, 92] Aggregation is an important factor influencing the contributions, and as such, the role of the particle emerges when aggregation is monitored with DLS.

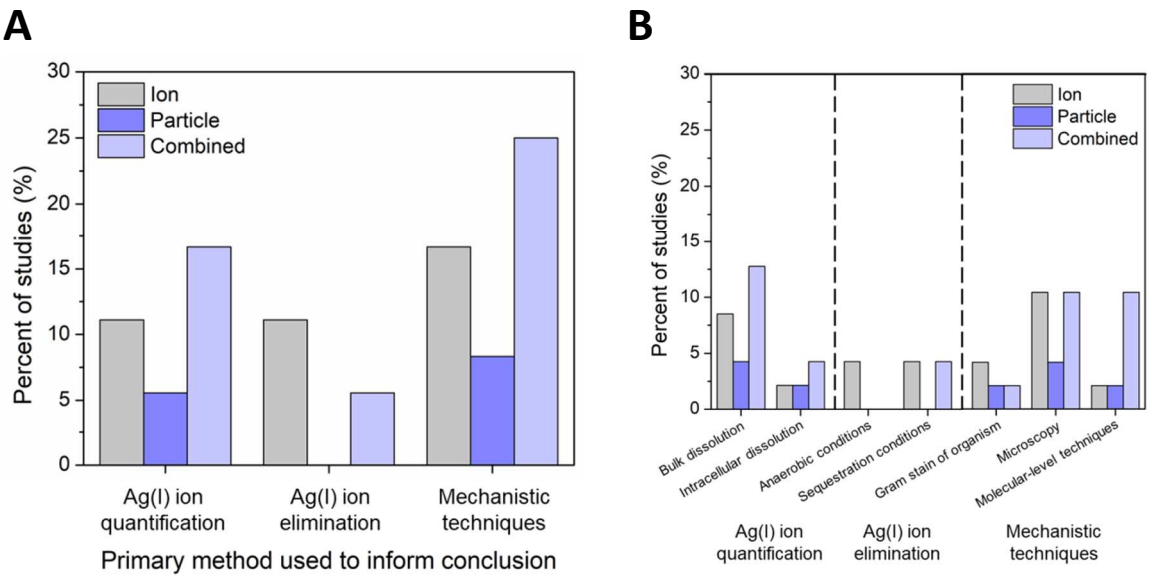


Figure 7. (A) Ion-only, particle-only, and combined ion-particle conclusions drawn from studies that used three main methods: Ag(I) ion quantification techniques,[23, 24, 57, 63, 64, 66, 68, 72, 73, 75, 77] Ag(I) ion elimination techniques,[27, 28, 33, 62, 82] or mechanistic techniques.[23, 24, 26, 31, 33, 62, 63, 65, 68, 71, 72, 76, 77, 82, 90-92] (B) Further break down of these primary methods into specific methods is presented (b). Note: some studies used multiple methods to aid in their conclusion drawn. Quantifying Ag(I) ion release and using molecular-level techniques provide support for particle-only and combined ion-particle mechanisms of antimicrobial activity while aerobic and sequestration conditions provide support for an ion-only mechanism.

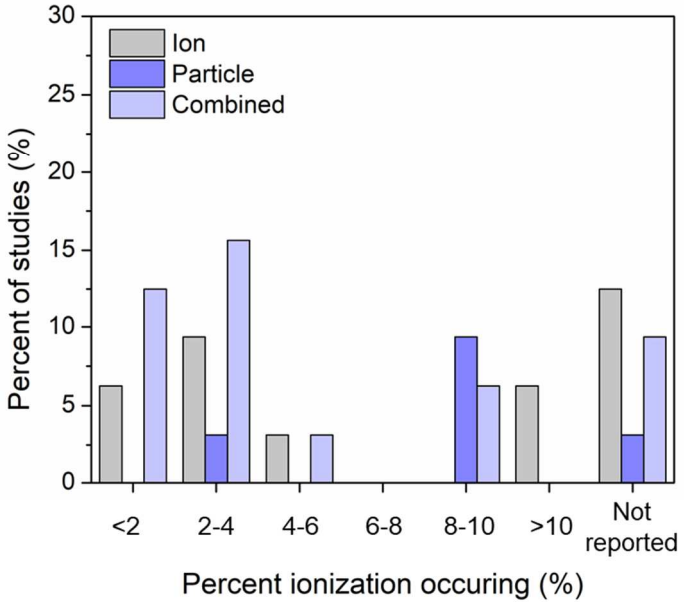


Figure 8. Ion-only, particle-only, and combined ion-particle conclusions drawn from studies that measured < 2%,[26, 33, 57, 62, 64, 67] 2-4%,[28, 38, 63, 66, 72, 73, 75, 77] 4-6%,[27, 76] 6-8%, 8-10%,[24, 31, 65, 70 38, 62, 65, 70, 73, 77, 91] > 10%,[68, 69] or did not measure[23, 71, 82, 86, 89-92] average ionization taking place in the AgNP system. The majority of studies across varying percentages of ionization occurring in the AgNP system concluded particle-only or combined ion-particle effects, suggesting that the mechanism of antimicrobial activity is not due to the particle releasing more Ag(I) ions.

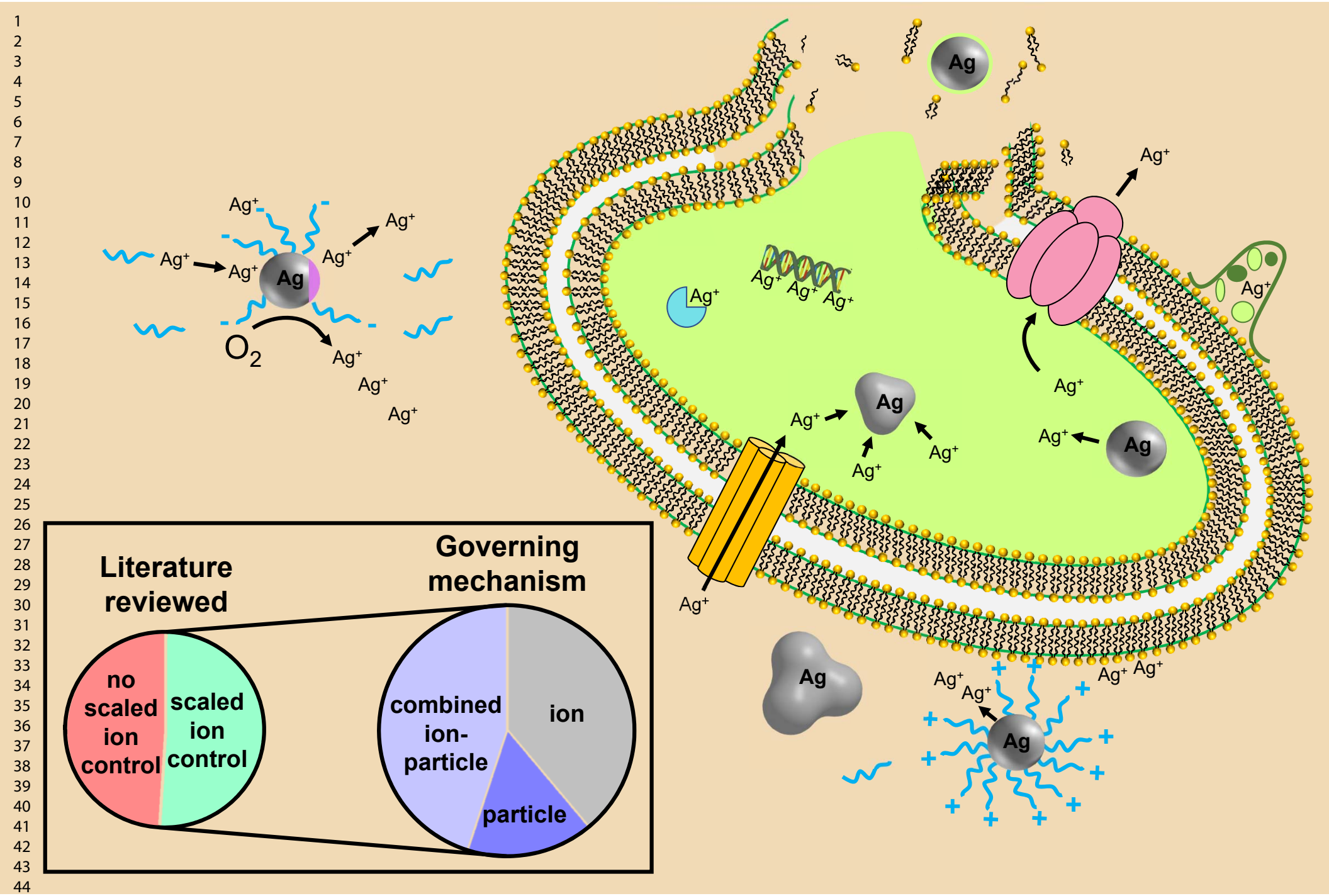
Table 2. Critical characterization parameters for future studies aiming to distinguish ion and particle contributions.

Criteria to Report	Possible Methods	Rationale
General NP characterization		
Initial NP concentration	ICP-MS or AES, UV-vis, or GF-AAS.	Critical for calculating theoretical ion release.
NP size	TEM in exposure media, pre- and post-exposure.	Necessary to quantify size effects and estimate surface area for calculating theoretical ion release.
NP shape	TEM in exposure media, pre- and post-exposure.	Necessary to estimate surface area for calculating theoretical ion release.
NP surface chemistry (e.g., ligand identities, sign and magnitude of charge, ligand density)	Zeta potential for sign and magnitude of charge in the experimental media pre- and post-exposure. NMR, TGA, IR-based methods (among others) for ligand density, pre- and post-exposure.	Changes in surface chemistry can result from exposure to the experimental system, which will influence ion release.
Method of NP synthesis and purification	Several; unique to NP.	Elucidate possible synthesis or purification artefacts causing different surface morphology and ion release.
NP aggregation	Monitor hydrodynamic diameter and zeta potential over duration of experiment and in experimental media using DLS. Monitor optical density over duration of experiment.	Confounds ion and particle contributions leading to differing conclusions drawn in studies (since aggregation can lead to non-nano sized particles).
Scaled ion control considerations*		
Bulk ion concentration	Quantify bulk ion concentration in broth and presence of organism over duration of experiment (e.g. using ion-selective electrodes, UV-vis spectroscopy, centrifugation and then quantification of ions in the	Completes the mass balance of silver in the system to inform delivery of a scaled ion dose and enable attribution of observed endpoints to the true exposed ion concentration.

	filtrate using elemental analysis techniques (e.g., ICP-MS or OES or GF-AAS).	
Intracellular ion concentration	E.g. Monitor intracellular ion concentration using a bioluminescent analogue of the model organism over the duration of the experiment.	
Media-complexed and cell-bound ion concentration	Method needed. An ideal method would be a standard assay that can isolate and quantify ions bound to media constituents or extracellular bacteria components.	
Ion concentration associated with laboratory materials used in experiments	Digest lab materials and measure ion content with ICP-MS/OES or GF-AAS.	

*There is a need to develop methodology for quantifying the distribution of silver in all components of the experimental system.

Abbreviations: DLS – dynamic light scattering; GF-AAS – graphite furnace atomic absorption spectroscopy; HDD – hydrodynamic diameter; ICP-MS or OES – inductively coupled plasma mass spectrometry or optical emission spectroscopy; NMR – nuclear magnetic resonance; SEM – scanning electron microscopy; TEM – transmission electron microscopy; UV-vis – ultraviolet-visible spectroscopy; XPS – x-ray photoelectron spectroscopy; XRD – x-ray diffraction


Ag
Ag+
O2
Literature reviewed
no scaled ion control
scaled ion control
Governing mechanism
combined ion-particle
ion
particle